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COMPARATIVE ANALYSIS OF ANTIOXIDANT ACTIVITY IN LEAVES OF DIFFERENT HOSTS INFECTED BY MISTLETOE (*VISCUM ALBUM* L. SUBSP. *ALBUM)*

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Abstract - Studies were performed at different sites in the major city of Lodz (Poland), where mistletoe is particularly abundant. The occurrence of total antioxidant enzyme activities such as superoxide dismutase (SOD), guaiacol peroxidase (POD) and the protein and phenol contents in leaves from different hosts infected by European mistletoe (*Viscum album* L. subsp. *album*) were studied. Some elements may vary in samples from the same mistletoe species on different host trees and species. The most important are changes in the concentration of proteins, including enzyme and stress proteins, in the leaves of trees infected by mistletoe, may indirectly indicate an interaction between host and parasite. There were no important differences in the soluble phenolic content after infection. The change in POD after infection was not significant in comparison to control plants. The SOD activity was significantly higher in leaves from infected trees in comparison to control plants. The obtained data suggest that the increase in SOD and protein content depends mainly on the host taxa. Moreover, we suggest that the main increase in SOD activities in leaves is a consequence of the stress oxidation that caused infection and development of the parasite on the host plant. Chemical analyses of mistletoe from a large sample of host species provide evidence that this parasite may be extremely tolerant to air pollution.

Key words: POD - guaiacol peroxidase, SOD - superoxide dismutase, host tree species, European mistletoe, Poland

INTRODUCTION

Viscum album (European mistletoe) is semi-parasitic, i.e. it depends on its respective host for water and mineral nutrition but is capable of producing carbohydrates by photosynthesis. It contains all of the pigments, chlorophyll a and b as well as carotenoids that are necessary for photosynthesis (Becker, 2000). Mistletoe has a much higher transpiration rate than its respective hosts. Schulze et al. (1984) found that mistletoe on pine trees has a more than 3-fold higher transpiration rate, calculated by leaf surface, compared to the pine tree. In a broad study on three continents, Ehleringer et al. (1985) investigated the transpiration and mineral nutrition of different mistletoes. They concluded that nitrogen supply is the limiting factor and that mistletoe transpiration is higher on hosts with low nitrogen content than on hosts with a higher nitrogen content in their xylem sap.

All mistletoes affect their respective host trees in many ways. They adversely affect the height and diameter growth, lower the vigor of the host, induce premature mortality, adversely affect the quality and quantity of wood produced, reduce fruiting of infected trees, and predispose trees to attacked by other

agents, such as insects or decay fungi. All these aspects also apply to *V. album* (Becker, 2000).

Viscum album is one of the most important biological stress sources for host plants, and mostly affects secondarily as nutrient and water stresses (Fischer, 1983; Schulze et al. 1984; Novacek, 1985; Marshall and Ehleringer, 1990; Pate et al. 1991; Türe et al. 2010).

Consequently, plants use certain strategies to overcome this stress condition. This adverse condition enhances the production of reactive oxygen species (ROS) which comprises both free radical ($O_2^{\bullet -}$, superoxide radicals; $OH^{\bullet}$, hydroxyl radical; $HO_2^{\bullet}$, perhydroxy radical and $RO^{\bullet}$, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen) (Mittler, 2002; Arora et al. 2002; Apel and Hirt, 2004; Gill and Tutaja, 2010). The antioxidant defense machinery protects plants against damage resulting from oxidative stress. Plants possess very efficient enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaiacol peroxidase, GOPX and glutathione-S- transferase, GST) and non-enzymatic (ascorbic acid, ASH; glutathione, GSH; phenolic compounds, alkaloids, non-protein amino acids and a-tocopherols) antioxidant defense systems which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging ROS. ROS also influence the expression of a number of genes and therefore control many processes like growth, cell cycle, programmed cell death (PCD), abiotic stress responses, pathogen defense, systemic signaling and development (Hiraga et al. 2001; Mittler, 2002; Apel and Hirt, 2004; Xiong et al. 2010; Gill and Tutaja, 2010). Superoxide dismutases (SODs), a group of metalloenzymes, are considered as the first defense against ROS, being responsible for the dismutation of O_2^- to H_2O_2 and O_2. CAT, APX, POD are enzymes that catalyze the conversion of H_2O_2 to water and O_2 (Gratao et al. 2005; Abedi and Pakniyat, 2010).

The interest in mistletoe among pharmacologists has arisen from the discovery of numerous pharmacologically active compounds obtained from the tissues of this semi-parasite: alkaloids, phenylpropanoids, triterpenes, polysaccharides, peptide compounds (including a cytotoxic viscotoxine), lectins, a number of amino acids, flavonoids, phytosterols (Fukunaga et al. 1987, 1988; Wagner and Jordan, 1988; Hariri et al. 1991; Richter and Popp, 1992; Łuczkiewicz et al. 2001; Haas et al. 2003). Alkaloids are nitrogenous compounds that may contribute to mistletoe's cytotoxicity (Franz, 1986). Various polysaccharides are thought to be involved in mistletoe's antineoplastic effects. Mistletoe extracts significantly reduced the DNA damaging effects of carcinogens (Bussing at al. 1996; Ochocka and Piotrowski, 2001). The parts used medicinally are the leaves and stems.

The division of *Viscum album* into subspecies was originally proposed based on slight morphological differences and was later confirmed by sequencing segments of the nuclear ribosomal DNA (internal transcribed spacer ITS) and noncoding cpDNA (Zuber and Widmer, 2009). Tree subspecies differ in their host specificity: *Viscum album* subsp. *album* grows on a wide variety of deciduous trees, *V. album* subsp. *abietis* is restricted to fir (*Abies* spp.), and *V. album* subsp. *austriacum* occurs mainly on pine (*Pinus* spp.). The fourth subsp., *V. album creticum*, is associated with a sole pine host, *Pinus halepensis* subsp. *brutia*, and occurs exclusively on the island of Crete (Barney et al. 1998; Böhling *et al.* 2003; Zuber, 2004; Zuber and Widmer, 2009).

Viscum album is distributed in Europe, with a native range up to Scandinavia in the north, Ukraine and Crimea in the east, and as an introduced species in Ireland and the northern part of Great Britain. In the east, it is known to occur in Middle Russia (W edge) and Belarus. Here, it is considered to be a decreasing species. It is scattered in the temperate zone of Asia, up to E China and Japan (Josephsen, 1993; Kubat 1997). It has also been introduced to North America (Hawksworth and Scharpf, 1986; Hawksworth et al. 1991).

The mistletoe in Poland is a generally common plant but with very uneven groupings of stations found from the Baltic coast as far as the lower parts of the Carpathian and Sudeten mountainsides. A literature survey revealed that mistletoe has been recorded on 164 taxa of trees and shrubs in Poland (Bojarczuk, 1971). As pointed out by Stypiński (1997), the vast majority of hosts for *V. album* subsp. *album* in Poland are *P. nigra*, *P.* × *canadensis* and *Malus domestica* varieties. Findings from different Polish towns and cities (i.e. Warsaw, Lodz, Torun, Poznan, Slupsk, Stetin) confirm that *Viscum album* L. subsp. *album* infected a similar range of alien host trees, with *Acer saccharinum*, *Populus x euramericana* and *Robinia pseudoacacia* among them (Markowski and Szmajda, 1971; Roniewicz, 1997; Zieiński, 1997; Kubus, 1998, Nienartowicz 1998; Jerzyk and Kluczyński, 2000; Zachwatowicz et al. 2008).

The activities of leaf peroxidase and dismutase are a reliable indicator of plant stress. Here we present for the first time comparative measurements of the dismutase and peroxidase activities of many pairs of different hosts and mistletoe species (Viscaceae) and different hosts. Studies were performed at different sites in Poland.

The main question asked was if the antioxidant activity of European mistletoe leaves differed from that of the leaves of their respective hosts. Specifically, this study addressed whetere there are any differences in: (i) peroxidase and dismutase activities between different hosts, and (ii) dismutase and peroxidase activities, soluble phenol and protein contents of the leaves of infected and non-infected host trees.

MATERIALS AND METHODS

Plants and sites

Leaf material was collected at the beginning of the vegetative season between 5 and 10 May 2012. In the May sampling, a representative sample of 5-10 leaves was collected from trees infected by mistletoe and control trees. Antioxidant activities in 11 samples from trees were analyzed. All individuals were assigned to *V. album* subspecies on the basis of their host trees. Based on this criterion, samples were assigned to *V. album* subsp. *album*, one to *V.* a. subsp. *austriacum*, and one to *V. album* subsp. *abietis*.

The populations sampled belong to distantly *related taxa* which differ in their host trees (Table 3). In this paper, "population" refers to the mistletoe plant sample from one host taxa from one investigated area. The sites of our plant material collections were from the city of Lodz (Fig. 3). The number of collected individuals was not the same for each population because different members of mistletoe were found on different host trees species.

For comparative analyses of the antioxidant activities and protein and phenol contents of plants infected by *Viscum album* subsp. *album*, leaf samples were taken from 11 populations from the city of Lodz. Lodz is the third largest city in Poland, located at 19°20' N/19°38' E in the central part of the country. It covers an area of 294.4 km^2 and in 2009 had about 740,000 inhabitants. The average annual precipitation in the period 1931-1998 ranged between 530 and 580 mm. The average annual temperature (1931-1998) ranged between 7.5 and 8.4°C. Elevation varies from 170 to 284.1 m a.s.l.

A wide variety of deciduous trees included individuals with mistletoes (infected trees) and individuals without any mistletoe (control trees found near each infected one). The deciduous leaves sampled on infected stems were all distal to the point of mistletoe attachment to the host stem. The infected stems and stems from the control trees had similar diameters (about 5 cm) and direction of sunlight exposure.

Enzyme assays

The tissue located up to 1 cm beneath the peel (0.5 g) was homogenized in a mortar and pestle with ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM ascorbate, 1 M NaCl, 1 mM EDTA and 1% polyvinylpyrrolidone. After centrifugation (20 000 x g; 15 min) the supernatant was used

to determine peroxidase and SOD activities and the protein content.

Assay of peroxidase (EC 1.11.1.7) activity

Peroxidase activity was assayed spectrophotometrically with guaiacol by measuring an increase in absorbance at 470 nM (ε= 26.6 $mM^{-1}cm^{-1}$) according to Maehly and Chance (1954). A mixture of 0.5 ml of the enzyme extract, 0.5 ml of 50 mM acetate buffer (pH 5.6), 0.5 ml of 20 mM guaiacol and 0.5 ml of 60 mM H_2O_2 was used. The enzyme activity was expressed in units (mmol tetraguaiacol min^{-1}) per g fresh weight.

Assay of superoxide dismutase (EC 1.15.1.1) activity

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Beauchamp and Fridovich (1971). A 3 ml reaction mixture contained 50 mM phosphate buffer at pH 7.8, 13 mM methionine (Sigma), 75 μM NBT, 2 μM riboflavin (Sigma), 0.1 mM EDTA (Sigma) and 0,020 ml enzyme extract. Riboflavin was added last and the tubes were placed 30 cm below two 15 W fluorescent lamps. The reaction was initiated by switching on the light and was allowed to run for 10 min. Switching off the light stopped the reaction and the tubes were covered with a black cloth. Controls were unilluminated tubes. The absorbances at 560 nm were read. The volume of extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

Assay of soluble phenol compounds

The soluble phenol contents were measured by the method of McCue et al. (2000). One gram of the tissue located up to 1 cm beneath the peel was homogenized in a mortar and pestle with ethanol (96%), and frozen at -20°C for 5 h, followed by centrifugation (20 000 x g; 15 min). Phenols were determined in the supernatants with Folin-Dennis reagent and 5% Na_2CO_3. The absorbance at 725 nm was measured. The soluble phenols were assessed by chlorogenic acid on 1g of fresh weight.

Assay of protein content

Protein was determined by the method of Bradford (1976) using bovine serum albumin (Sigma) as a standard.

Statistical analysis

All results presented are the means of eight independent analyses (n=10). Sample variability was given as the standard deviation of the mean. The significance of differences between mean values was determined by a non-parametric Mann-Whitney rank sum Test (STATISTICA Software edition 1998). Differences between examined and control groups were considered significant at $P \leq 0.05$.

RESULTS

Studies of the control leaves and those infected by mistletoe revealed a significantly higher content ($P < 0.01$) of leaf protein in most species of the infected trees than in the control trees (Fig 1. A). This is 108-228% higher in respect to control trees. Only in the case of *Malus domestica* (Fig 1. A (H)) was there no certified, amplified content after infection. Significant differences in the level of protein content were revealed between the studied host trees. The mean values of protein concentration in *Acer saccharinum* host trees was almost twice as high as other host taxa.

The soluble phenolic levels in the control leaves was 7-49 ± 0.9-3.4 (mg/fresh weight) and in leaves infected with mistletoe it was 13-4 ± 1.1-5.6 (mg/fresh weight) showing no important differences in the soluble phenol compounds content after infection (Fig. 1 B). As with protein, there were similar differences between the taxa of the studied trees. It is important that the same species of trees demonstrated similar concentrations of phenol compounds (Fig. 1 (B) – A, B, C, D – *A. saccharinum*).

In all samples, there were insignificant changes in POD after infection in comparison to the control (Fig. 1 A). In this case, activity was insignificantly

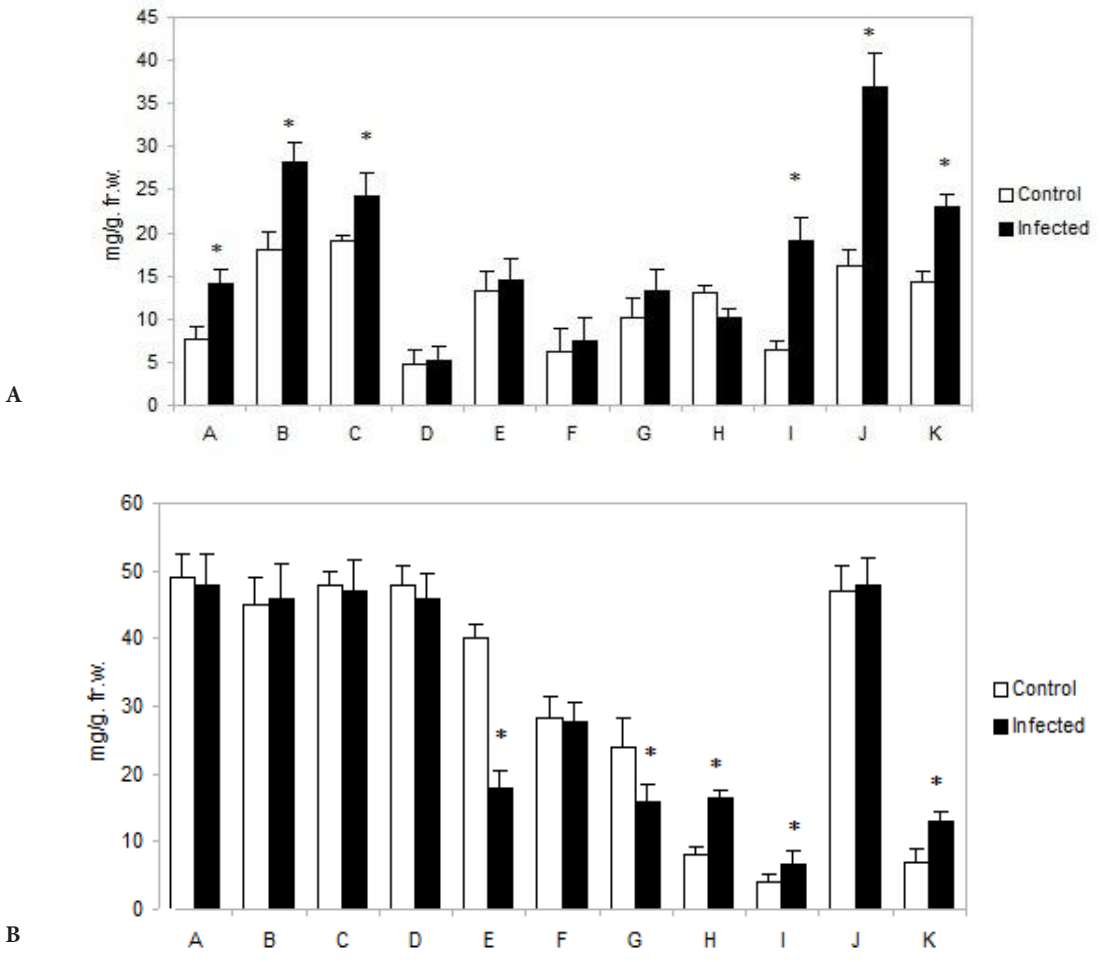

Fig. 1. Protein (A) and soluble phenolic (B) contents in the leaves of different taxa of tree in control and infected by mistletoes. (A, B, C, D - *Acer saccharinum*, E - *Populus* 'NE 194', F, G - *Malus purpurea*, H – *Malus domestica*, I - *Sorbus aucuparia*, J - *Acer pseudoplatanus*, K - *Crataegus oxyacantha*). Vertical bars indicate ± S.D., n = 10. * P≤0.05.

lower in plants infected by mistletoe. POD activity in the same host tree was different. In leaf extracts, the highest POD activity was found in *Populus* 'NE 194', 6.9 ± 0.22 U/g fresh weigh, while the lowest value was 0.31 ± 0.11 U/g fresh weigh for *A. saccharinum*.

In one of the 11 studied samples the lower SOD activity after infection was significant and in the remaining 10 samples it was statistically higher in comparison to control plants (66-256 U/g fresh weigh, and 76-305 U/g fresh weigh, respectively) (Fig. 2 B). The highest level of SOD was detected in leaf extracts of cultivar *Populus 'NE 194'*, and the leaf extracts of *Crataegus oxyacantha* host had the lowest level. Host trees vary greatly in their physiology. Some elements may vary by one or two orders of magnitude in samples from the same mistletoe species on different host trees.

DISCUSSION

Here we present for the first time comparative measurements of the activity of leaf peroxidase and SOD of many pairs of European mistletoe and different hosts. Strong oxidation reactions seemed to occur in the junction between host tree and mistletoe. It is widely

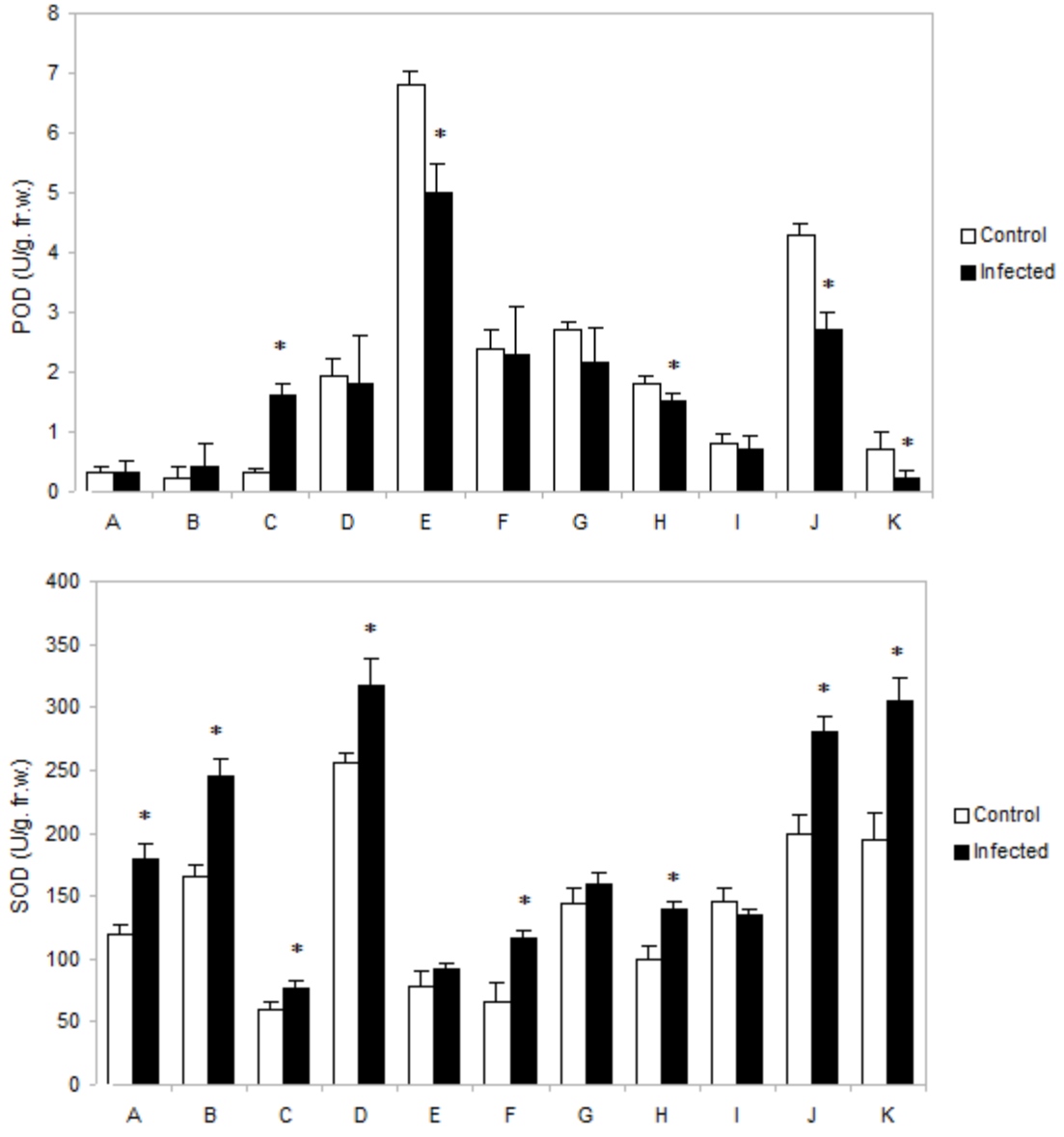

Fig. 2. Guaiacol peroxidase (POD) activity (A) and superoxide dismutase (SOD) activity (B) in the leaves of different taxa of trees in control and after infection by mistletoe. (A, B, C, D - *Acer saccharinum*, E - *Populus* 'NE 194', F, G - *Malus purpurea*, H – *Malus domestica*, I - *Sorbus aucuparia*, J - *Acer pseudoplatanus*, K - *Crataegus oxyacantha*). Vertical bars indicate ± S.D., n = 10. * $P \leq 0.05$.

appreciated that the chemical composition of mistletoe is not stable and depends not only on biosynthesis but also on the type of host plant and growth conditions, such as ambient temperature, carbon dioxide concentration and season of the year (Stypiński 1997; Łuczkiewicz et al. 2001). Therefore, the differences in antioxidant activity between the leaves and stems of the mistletoe harvested from different trees can be attributed to environmental factors such as season, climate and temperature which can significantly affect the accumulation of the antioxidant components in the plant tissue (Vicas et al., 2008).

Table 1. Protein (A) and soluble phenolic (B) contents in the leaves of different taxa of tree in control and infected by mistletoes. n = 10. * *P≤0.05.*

No.	Protein		Phenols	
	Control	Infected	Control	Infected
A	7.7±1.4	14.1±1.6 *	49.1±3.4	48.0±4.7
B	18.1±2	28.1±2.3 *	45.0±4.1	45.9±5.1
C	19.0±0.8	24.2±2.7 *	48.0±1.8	47.0±4.5
D	4.7±1.7	5.1±1.8	48.0±2.8	46.0±3.7
E	13.2±2.3	14.6±2.5	40.0±2.2	18.0±2.5 *
F	6.3±2.6	7.4±2.6	28.3±3.2	27.8±2.8
G	10.2±2.3	13.2±2.8	24.0±4.2	16.0±2.6 *
H	13.0±0.9	10.1±1.1	8.0±1.1	16.5±1.1 *
I	6.5±0.9	19.0±2.8 *	4.1±1.0	6.5±2.1
J	16.2±1.8	37.0±3.8 *	47.0±3.8	48.0±3.8
K	14.3±1.3	23.0±1.4 *	7.0±1.8	13.0±1.4

Table 2. Guaiacol peroxidase (POD) activity (A) and superoxide dismutase (SOD) activity (B) in the leaves of different taxa of trees in control and after infection by mistletoe. n = 10. * *P≤0.05.*

No.	Protein		Phenolics	
	Control	Infected	Control	Infected
A	0.31±0.1	0.33±0.2	120±7.1	180±12 *
B	0.23±0.2	0.41±0.3	165±9.2	245±14 *
C	0.32±0.07	1.6±0.2	60±6	76±7 *
D	1.92±0.31	1.82±0.8	256±8	318±20 *
E	6.8±0.22	5.0±0.5 *	78±12	92±4
F	2.4±0.31	2.3±0.9	66±15	117±5 *
G	2.7±0.15	2.15±0.6	144±13	160±8
H	1.8±0.14	1.51±0.15	100±11	140±6 *
I	0.82±0.13	0.71±0.21	145±15	135±4
J	4.3±0.2	2.71±0.3 *	200±15	280±12 *
K	0,7±0,3	0,24±0,12	195±21	305±19 *

Host conditions may affect in various ways the mistletoe parasite. Neighboring host trees of the same species frequently show very different infestations by parasites. The failure to establish mistletoe may be due to the different attractiveness of neighboring host trees to dispersers or owing to differences in seed attachment and penetration of the bark by the germinant. As mistletoe seeds must strongly adhere to the bark to allow the germinant to penetrate the bark, small differences in the physical or chemical properties of the bark obviously can make a big difference in establishment. In dwarf mistletoes (*Arceuthobium*), the biochemical properties of the host's xylem and phloem have a direct influence on the physiological performance and ultimately the survival of the mistletoe (Linhart et al. 2003). Although the cumulative impacts of a long infestation and large mistletoe load can result in severe pathogenic effects on the host, the relationship between host conditions and mistletoe performance can vary by situation and over time (Glatzel and Geils, 2009).

These hosts vary greatly in their mineral composition and physiology, and they include hosts that have a high resistance to the harmful effects of city environment. For example, Silver maple (*Acer saccharinum*) is a relatively fast-growing tree that is highly adaptable, although it has higher sunlight requirements than other maples. This species is highly tolerant to a wide range of soils, drought, seasonal flooding and urban conditions; it is therefore frequently planted next to streets (Day et al. 2000).

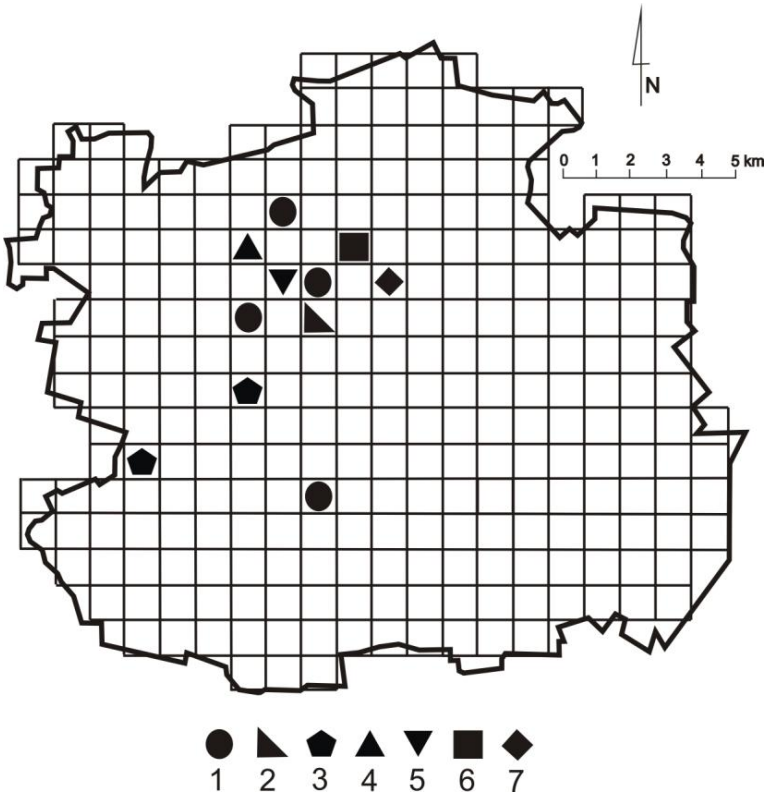

Figure 3. A map showing locations of the studied sites with mistletoe parasitizing deciduous tree populations on the background of 1-km squares in the city of Lodz
1 – *Acer saccharinum*, 2 – *Populus* 'NE 194', 3 – *Malus purpurea,* 4 – *M. domestica,* 5 – *Sorbus aucuparia,* 6 – *Acer pseudoplatanus,* 7 – *Crataegus oxyacantha*.

The pattern of peroxidase and dismutase activities may be different for certain samples from the same mistletoe species on different host trees and species. A great number of researchers have stated that the first symptom of a plant's contact with parasites are peroxidase activity changes, but this does not signify that activity should increase in tissues infected by a pathogen (Bestwick et al., 1998). After a few minutes the plant's react by an oxidative burst. The oxidative burst in plants is a response to pathogen infection (Wojtaszek, 1997). An oxidative burst frequently induces an increase in SOD activity. Most plant tissues are able to produce H_2O_2, either constitutively or under stress situations, and the result is not always net H_2O_2 accumulation (Herńadez et al. 2001: Mittler et al. 2004).The identification of common strategies involving antioxidant systems is further hindered by the fact that only some of the parameters of the ROS-scavenging machinery were analyzed in each study. Moreover, the possibility of species-specific responses further complicates the emerging picture (De Gara et al., 2003). We suggest that the increased SOD activity in the studied plants might be decisive for the stable state of homeostasis in plant tissue permanently exposed to contact with mistletoe (Foyer and Noctor., 2005). It is hard to explain simply without referring to genetic methods, if increased SOD activity is a plant's response to a pathogen or, as is equally possible, whether it originates from the parasite that defends itself from ROS produced by the plant.

The accumulation of soluble phenolics accompanies the oxidative burst and phenolic oxidation

(Baker el al., 2005). It is interesting that there were no significant changes in the soluble phenol compounds concentration between infected and control leaves. In this case, we can assume that it is caused because of not fully-grown leaves. An accumulation of phenolic compounds concerns mostly old, fully-grown leaves. We did not aim to demonstrate essential changes in POD activities in leaves after infection but it does not mean that in the majority, insignificant negative changes that were detected at the beginning of vegetation season are the beginning of changes that will occur in future – this is an aim for further studies. The most important changes are those in protein concentration, which may indirectly indicate an interaction between host and mistletoe. Marked protein may be enzymes as well as stress proteins. The most important conclusion from this study is the significant change in SOD observed in most of the studied trees infected by mistletoe.

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